Design and Development of Alginate Based Drop on Demand Setup for Bio-Fabrication

-DATE MAHENDRA DAGADU-

A Dissertation Submitted to

Indian Institute of Technology Hyderabad

In Partial Fulfillment of the Requirements for

The Degree of Master of Technology

Department of Mechanical and Aerospace Engineering

June, 2016

Declaration

I declare that this written submission represents my ideas in my own words, and where others' ideas or words have been included, I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that any violation of the above will be a cause for disciplinary action by the Institute and can also evoke penal action from the sources that have thus not been properly cited, or from whom proper permission has not been taken when needed.

_____

(Signature)

Date Mahendra Dagadu

(Student Name)

ME14MTECH11014

(Roll No)

Approval Sheet

This thesis entitled **Design and Development of Alginate-Based Drop on Demand Setup for Bio-Fabrication** by **Date Mahendra Dagadu** is approved for the degree of Master of Technology from IIT Hyderabad.

Dr. Abhay Sharma.
Dept. of Mechanical and Aerospace Engineering
IIT Hyderabad
Examiner

Dr. S Suryakumar
Dept. of Mechanical and Aerospace Engineering
IIT Hyderabad
Adviser

Dr. Subha Narayan Rath
Dept. of Biomedical Engineering
IIT Hyderabad
Chairman

Acknowledgements

It is a privilege for me to write the few lines in acknowledgement of those who enabled me to carry out the project work. I express my deep gratitude to my thesis adviser **Dr. S Suryakumar**, Associate Professor, Department of Mechanical and Aerospace Engineering, Indian Institute of Technology Hyderabad for his guidance, help and support to my project. His affability boosted my moral towards the working of the project. I express my deep and sincere gratitude, regards and thanks to him for his guidance, valuable suggestions and continuous encouragement at all the stages of my project work. His wide knowledge and logical way of thinking have been of great value for me.

It was a great pleasure for me to be a part of the Department of Mechanical and Aerospace engineering, IIT Hyderabad and I would like to thank all the staff members and my friends for helping me in all stages of my work.

Dedicated to

This little study and modified setup is dedicated to people working in the field of Additive Manufacturing for making this world beautiful and easy to live.

Abstract

With advancement of Tissue engineering field, possibility of the regeneration of the failed organs, injured tissues became realistic. The combination of living cells and bioactive materials, the developed tissue engineered construct that can replace the damaged or lost tissue either by laboratory-grown construct or by allowing the construct to grow inside the body. Additive Manufacturing method, which is one of the cause of advancement in Tissue Engineering, which allows the fabrication of the artificial construct with fulfilling its basic criterion, such as highly porous interconnected structure has possible because of advancement of the bio materials. Additive Manufacturing which has ability to add material at desired location to make a component is best suited for creating such a scheme for cell culture. It has a unique ability to precisely control of the matrix architecture like size, shape, interconnectivity, branching, geometry and orientation.

This study is consisting of the development of the new setup for economical fabrication of the alginate based scaffold. To achieve this, the hardware of economical FDM printer was modified into Drop on Demand (DoD) based setup. In the setup development study, the deposition of Calcium chloride ($CaCl_2$) on the sodium alginate (Na-Alg) has been tried to observe the swelling effects. To go for the 3D structure of the scaffold, in further studies, the extrusion of Na-Alg was done over the $CaCl_2$. And with this change further studies were done to develop the setup. The parameters which affect the most; concentrations of the solutions, the deposition speeds, indirect control of flow rates, were studied and tried to fix some range for them for the best outcomes. To address the difficulties with the multi-layer deposition special mechanism was developed and attached to the bed for multi-layer deposition for development of the scaffold.

Nomenclature

3D	-	3 dimensional
AM	-	Additive manufacturing
$CaCl_2$	-	Calcium chloride
CAD	-	Computer aided design
CO_2	-	Carbon dioxide
CT	-	Computer tomography
DoD	-	Drop on demand
FDM	-	Fused deposition modeling
HA		Hydroxyapatite
MRI	-	Magnetic resonance imaging
Na-Alg	-	Sodium alginate
PCL	-	Polycaprolactone
PLA	-	Polylactic acid
PLGA	-	Poly(lactic-co-glycolic acid)
PLLA	-	Poly-L-lactide
RP	-	Rapid prototyping
SFF	-	Solid freeform fabrication
SLA	-	Stereolithography
SLS	-	Selective laser sintering
TE	-	Tissue Engineering
TEC	-	Tissue engineered construct
TPP	-	Two-photon polymerization
TCP	-	Tri-calcium phosphate

Contents

List of Figures

List of Tables

Chapter 1

Introduction

"The application of principles and methods of engineering and life sciences toward the fundamental understanding of structure-function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain or improve tissue function", is how Tissue Engineering (TE) is defined by National Science Foundation [1]. TE is aiming to replace the damaged tissue with engineered tissues as well as to reduce the gap between the organs needed for transplantation and available numbers of organs for transplantation. The underlying concept or principle of tissue engineering is the belief that cells can be isolated from a patient, and its population then expanded in a cell culture and seeded onto a carrier called scaffold or Tissue Engineered Construct (TEC) [2]. Figure 1.1 illustrates the basic principle of the Tissue Engineering. In Tissue Engineering, the most dominant and commonly used method is printing of scaffold/ matrix and combing it with living cells for replacing, regenerating or repairing of the original tissue.

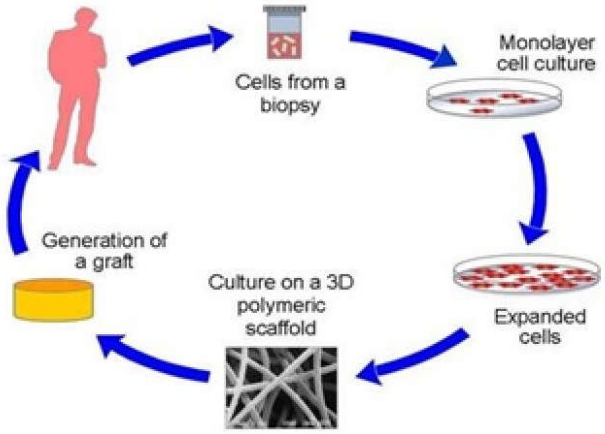

Figure 1.1 Principle of the tissue engineering [3]

1.1 Tissue Engineering Construct/ Scaffold

Tissue Engineering Construct or Scaffold is a extracellular matrix whose functions are to act as template for tissue formation, to provide the structural as well as the biochemical support, facilitate the migration of the living cells of the tissues [2]. Scaffolds can be broadly classified into two types viz., Hard scaffold and Soft scaffold. Hard scaffolds (Figure 1.2) are nothing but the bones of the body while soft scaffolds (Figure 1.3), include cartilage, bladder, blood vessels, muscles etc. These scaffolds which are cell seeded, are cultured in vitro environment for synthetization so that can be implanted later into the body at infected or injured site, or they can

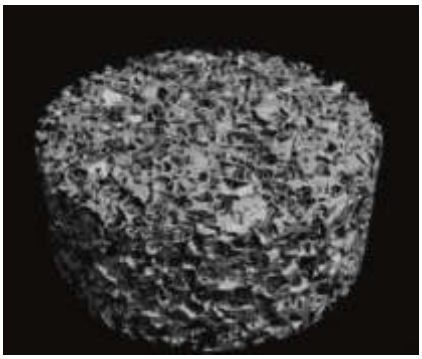

Figure 1.2 Hard Tissue Engineered Construct [5]

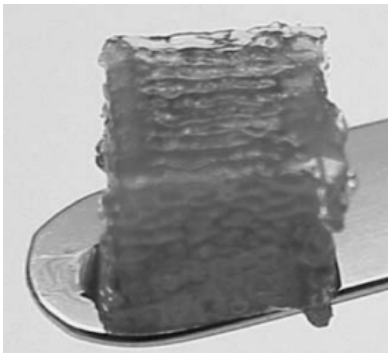

Figure 1.3 Soft Tissue Engineered Construct [5]

be directly implanted into body for regeneration of organ or tissue using own body system in vivo.

1.2 Requirements of Scaffolds

There are a number of biomaterials and manufacturing methods used for producing the scaffolds that are used for tissue regenerations. While there are many important factors that need to be consider while checking the suitability for scaffold, biocompatibility, biodegradability, mechanical properties, architecture and manufacturability are some the important ones that are to be taken care of, regardless of the material and fabrication methods used.

1.2.1 Biocompatibility

The scaffold must be biocompatible. The cell must be adhering to it, must function normally and able to migrate through the scaffold. After the implantation it shall evoke minimum immune reaction in order to prevent reduction in healing or rejection of implanted/ regenerated tissue by body [2].

1.2.2 Biodegradability

The aim of TE is that implanted scaffold must be replaced by own body's cell system over a time. Hence the scaffold or TEC must be also bio-degradable, to allow the own body cell system to produce their own extracellular matrix [3]. The degradation byproduct should not be toxic and can be carried out of body without affecting the functions as well as healing.

1.2.3 Mechanical properties

Mechanical properties like strength, stiffness etc. of the scaffolds must be similar to that of the implanted part, ideally and from practical view also. The implanted TEC must have integrity to function from implanting till complete

remodeling of process [2]. Once the artificial scaffold has to undergo degradation it would have to retain its properties integrated with newly developing natural matrix.

1.2.4 Scaffold architecture

Scaffold architecture is having very critical importance in tissue engineering. TEC must have highly porous and interconnected structure as shown in Figure 1.4, so that sufficient diffusion of nutrients within the scaffold and the extracellular matrix can happen. Also, highly porous structure ensures the cellular penetration. The interconnected porous structure is allowing the diffusion of degradation byproduct and other waste products out of the construct, so that they shall be able to exit the body without interfering with any other organs and surrounding tissues [4].

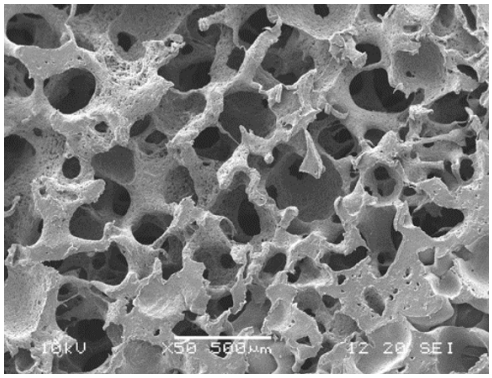

Figure 1.4 Porous architecture of the scaffold [7]

1.3 Scaffold Fabrication

Research on the fabrication of TE construct has been going on for more than three decades. There are conventional techniques like solvent casting, membrane lamination, fiber bonding etc. used. But with these conventional methods, it is difficult to fulfill the requirements of the scaffold. The Solid Freeform Fabrication (SFF) or Additive Manufacturing (AM) methods were introduced in 90's for scaffold printing and gained a lot of popularity in the TE field due to their flexibility. In these

methods, the TEC is made by adding the biomaterials layer by layer with computer control [5].

There are numerous biomaterials available along with the manufacturing techniques. The scaffold, in order to become clinically and commercially viable, should be cost effective for the given material as well as the fabrication methodology used should be scaled up from one at a time to small batch production at lower cost [6].

1.4 Problem Definition

The motive of this study is to use advantages of the Additive Manufacturing for constructing mainly the soft scaffolds. Developing hard scaffold with this technology is comparably simple as it is easy to have control over the semi-solid extrusion material.

This work attempts to design and develop a new the Drop on Demand (DoD) method of liquid jetting for the economical fabrication soft tissue with the help of open source hardware and software. The initial studies are based on polymerization of sodium-alginate (Na-Alg) with the help of polymerizing agent calcium chloride ($CaCl_2$). The concentrations of the Na-Alg & $CaCl_2$ on the polymerized grain, their size and shapes. Later the study is extended to the single-layer deposition with this new methodology and further extended to possible ways for multi-layer deposition.

1.5 Outline of the Thesis

This report is divided into the five chapters and the work is arranged as follows:

Chapter 1 introduces the background of TE, Scaffold or TEC, requirements of TEC, manufacturing methods.

Chapter 2 presents the literature survey, including the various technologies used for the scaffold fabrication, different biomaterials and methods used for

construction of scaffolds, their performance in vitro & in vivo and various parameters affecting this performance.

Chapter 3 introduces the machines and software used for the study, their specifications, modifications and experimental processes.

Chapter 4 presents the various studies done for single-layer deposition, while the same for multiple layer deposition are presented in *Chapter 5*.

Chapter 6 summarizes the work and the future scope.

Chapter 2

Literature Survey

2.1 Overview

The available literature on the subject of TE is presented in this chapter and is divided into the following parts. The first part consists of a different technologies used for Tissue Engineering Construct, their advantages and disadvantages. Second part discusses the different additive manufacturing technologies used for the scaffold fabrication. Third part contains the biomaterials used for both hard scaffold fabrication as well as soft scaffold fabrications, forth part contains the different Drop On Demand methods used for soft scaffold fabrication and last part contains the selection criteria of biomaterials for the drop on demand techniques.

2.2 Conventional Techniques for Scaffold Fabrications

Langer et al. first studied and introduced scaffold based tissue engineering concepts which involve the use of combinations of viable cells, biomolecules and a structural scaffold combined into a TEC to promote the repair and regeneration of tissues [5]. Different scaffold fabrication methodologies/techniques utilizing several synthetic and natural polymers, including solvent casting, membrane lamination, particulate leaching, melt molding, fiber bonding, gas foaming, phase separation,

freeze drying, electrospinning, fiber meshing and additive manufacturing. Some of these techniques are discussed below.

Solvent Casting

This is the simple and inexpensive technique, based on the evaporation of one of the two solvent, does not required large equipment. There are two methods, one is adding the polymeric solution into a mold and another is to dip the mold into polymeric solution. Figure 2.1 shows, the micrograph of scaffold fabricated by solvent casting. This technique is having disadvantage like, the toxic solvent contaminates the protein, can affect other solvents, may also retain some of the toxicity. To solve these issues scaffolds used to dry completely by vacuum process. But, this process is time consuming and to overcome these problems some researchers have combined it with particulate leaching techniques [7] [8].

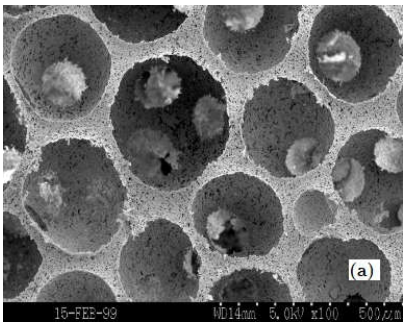

Figure 2.1 SEM micrographs of poly(α-hydroxy acids) scaffolds prepared by solvent casting [7]

Particulate-Leaching Techniques

Porogens which are nothing but salt, wax or sugars, used to create channels or pores. In this technique salt is grinded into particles and those small particles are poured into a mold and filled with the porogen. A polymer solution is casted then into mold. The salt crystals are leached away using water to form the pores of the scaffold, after the evaporation of the solvent. In Figure 2.2 PLGA scaffold prepared with paraffin spheres by particulate leaching technique [7] is shown. The advantage

this technique is it requires less amount of polymer for fabrication of the scaffold. However, variables such as inter-pore connections and pore shapes cannot be

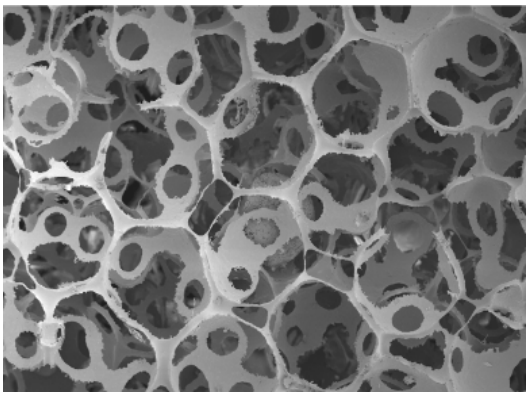

Figure 2.2 PLGA scaffold prepared with paraffin spheres by particulate leaching technique [7]

controlled [9].

Membrane Lamination

Membrane lamination is similar to solid free from technique used for fabrication of 3-D foam scaffolds with precise anatomical shapes. Membrane lamination is done by solvent casting and particle leaching and introducing proteins as well as peptide in layers during the fabrication. The membranes are then soaked in solvent and then stacked up in 3D with continuous pore structure. The properties of the final three dimensional scaffolds are similar to those of the individual membrane. It is possible to use the computer assisted modeling with this technique. disadvantages of this technique are, there is poor pore interconnectivity, it is a time consuming process because of use of thin membrane [10].

Gas Foaming

The gas foaming methods do not require organic solvents and high temperature, like other methods. It uses high pressure CO_2 gas to fabricate TEC. The porous structure of the construct is depending upon the how much CO_2 dissolved in the fabrication polymer. This dissolved CO_2 starts to become unstable and to

minimize the energy of the molecules of CO_2 starts becoming cluster which results into starting of pore nucleation. Figure 2.3 Schematic of the gas foaming process. Lyophilized growth factors mixed with PLGA particles and NaCl are subject to high pressure CO2 incubation [11]. The pores result into expansion of polymer and decrease in density. 3D porous structure of the scaffolds is formed after completion of this foaming process [11].

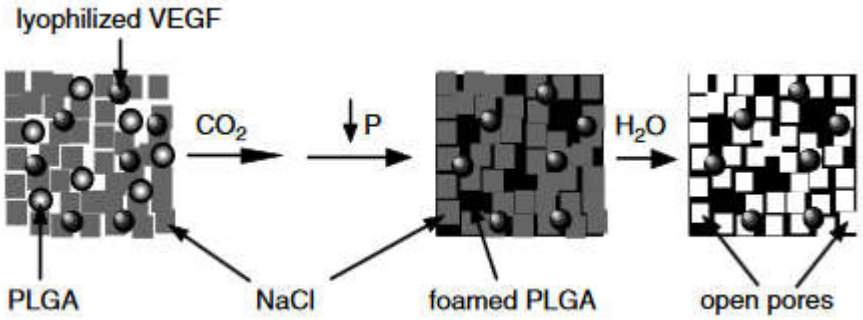

Figure 2.3 Schematic of the gas foaming process. Lyophilized growth factors mixed with PLGA particles and NaCl are subject to high pressure CO_2 incubation [11]

Phase Separation

This technique requires the change in the temperature to separate the two phases of the polymer viz, polymer lean phase and polymer rich phase. Fabrication polymer is dissolved in the naphthalene or in the phenol, after this the biomolecules

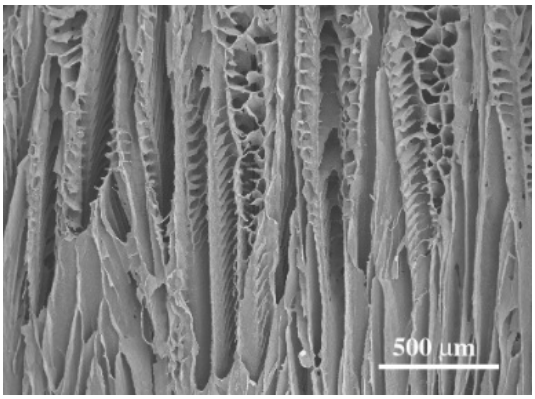

Figure 2.4 SEM micrographs of porous PLLA scaffolds prepared in benzene or dioxane solutions using a phase-separation technique [16]

are added into this solution and the temperature is lowered to separate the liquid-liquid phases of the polymers which is followed by the quenching to form the solid of two structure. The solvent is then removed by the sublimation, evaporation and the extraction. Advantages of this technique is that it can be easily combine with the other fabrication technologies [12] [13].

Additive Manufacturing (AM)

Additive Manufacturing is also known as solid freeform fabrication (SFF) technique. It is the most advanced, computer controlled scaffold fabrication technique. In this technique the three dimensional object can be produced by adding the material in layers from bottom with moving up layer by layer. Figure 2.5 shows how AM technique is used to create tissue engineering construct. The major advantages of this technique are it can integrate with different imaging technique to produce the customized sized and shaped scaffold as per particular application for individual patient, it is easy to control the architecture which includes the shape, size, porosity, interconnectivity of pores, orientation and geometry [14]. This technique is having ability to control degradation kinetics of scaffolds, mechanical property, biological effects and yielding biomimetic structure which vary in design as well as material composition [10]. Because of these advantages this technique gains the popularity compare to other techniques. while there is limitation with this technique is its lower resolution.

These are some of the popular conventional techniques used in the bio fabrication of the scaffold. While after development of the additive manufacturing, because of the advantages they provide, other techniques were getting outlaying, where some of them are still used in combination with them [15].

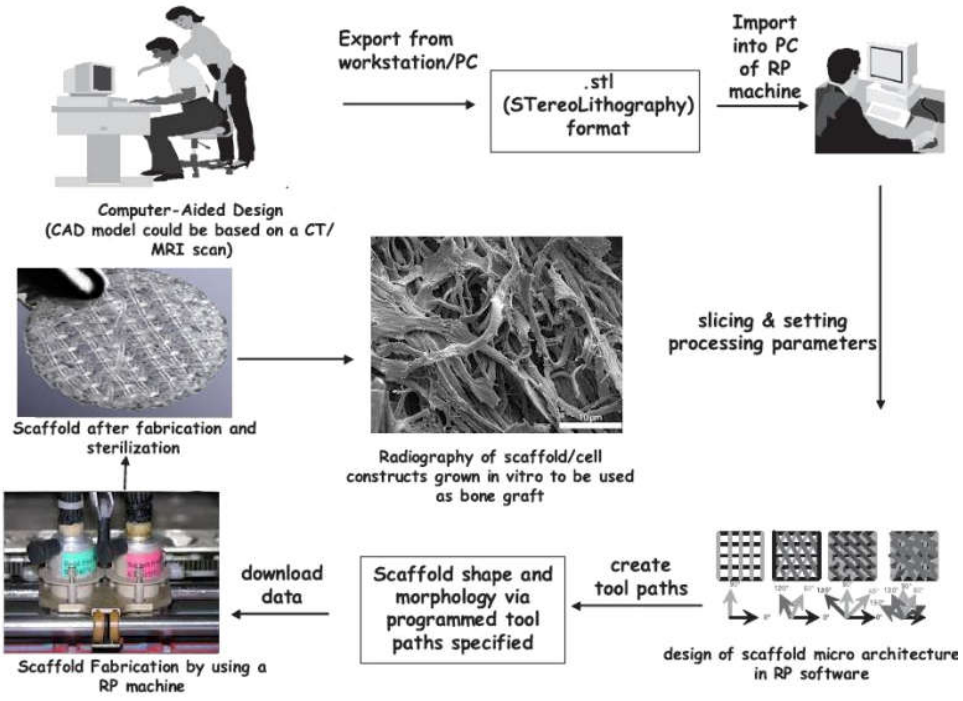

Figure 2.5 Flowchart of the scaffold fabrication with AM technique [5]

2.3 Additive Manufacturing Techniques for Scaffold Fabrications

Different additive manufacturing techniques that have been developed and used in TE, can be broadly classified as the Laser based solidification techniques and nozzle based techniques [16].

Stereolithography (SLA), utilizes a laser beam or other light sources for selectively photo-polymerization of the photosensitive liquid polymers layer by layer, Figure 2.6 shows the schematic of the SLA. This method has be used to construct the scaffold using photo-polymerizable polymers, where the laser or UV light curable polymers in liquid form are cross linked selectively by emitting the light source [17], [18]. Two-Photon Polymerization (TPP) is another photo-polymerization AM technique which is having the better resolution [19]. With the SLA technique the mechanical strength and accuracy of the product is good but the major limitation of the method is, it will applicable to photo curable polymers only [19].

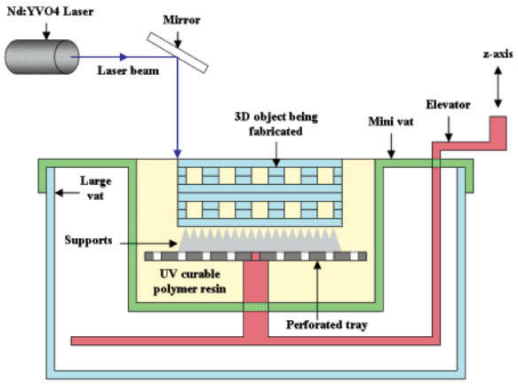

Figure 2.6 Schematic of the stereolithography system [47]

Selective Laser Sintering (SLS), laser-based AM technology, in which the laser beam scan selectively over the powder bed according to cross section. This results in the fusing powder particles together. SLS is having large range of materials; ceramics, metals as well as some polymers like Polycaprolactone (PCL), Poly-ε-caprolactone, hydroxyapatite, carbonated hydroxyapatite can be used [20]. Metals used in the SLS are Copper (Cu), Cobalt (Co), Iron (Fe), Steels, Co-alloys, Lead (Pb), Titanium (Ti) etc, in the powder form, Ceramics used are Aluminum Oxides, Zinc Oxides, Silicon tri-oxide, Titanium Oxides etc. SLS fits for fabrication of complex geometries of TE,

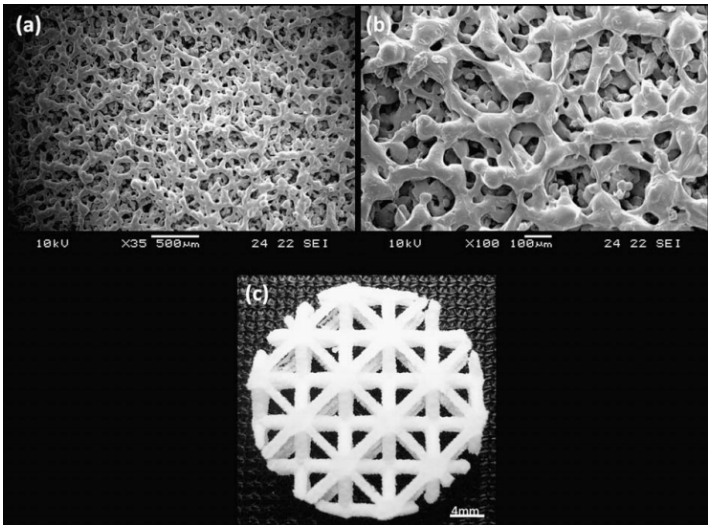

Figure 2.7 Morphology of PCL particles sintered at 30 W laser power (a) SEM at 35X magnification; (b) SEM at 100X magnification (c) the macrostructure of the sintered scaffold [20]

and commonly used for the fabrication of the artificial bones. Figure 2.7 shows the morphology of the Polycaprolactone sintered structure. Because of use of laser beam energy inputs required for the SLS are high, also it is difficult to control the porosity of the construct with it [21].

Fused Deposition Modeling (FDM), the widely used nozzle based AM technology for hard scaffold fabrication, uses the thermoplastic polymers. In FDM the thermoplastic is heated to the semisolid state and extruded in the form of very thin wire through the nozzle is deposited precisely on to the bed in the form of lay down pattern, different laydown patterns are shown in the Figure 2.8. After completion of layer, Z axis is moved and next layer printed on it which would be in semi-solid state getting fused with previous layer because of heat available into it [22]. With FDM, it easy to fabricate the processed file or digital data obtained from the imaging techniques such as computer tomography (CT) or magnetic resonance imaging (MRI). Major advantage FDM provides is low cost fabrication but the materials available are not very large in range. Also, in bio fabrication view the temperatures used for the heating the extrusion thermoplastic polymers are high and living cells cannot be surviving at this elevated temperature. Using the same principle as of FDM, 3D bio-plotting technology developed by which the fabrication of soft scaffold can be made, also this uses variety of polymers as well as pastes, plastisol, solutions and reactive oligomers [21].

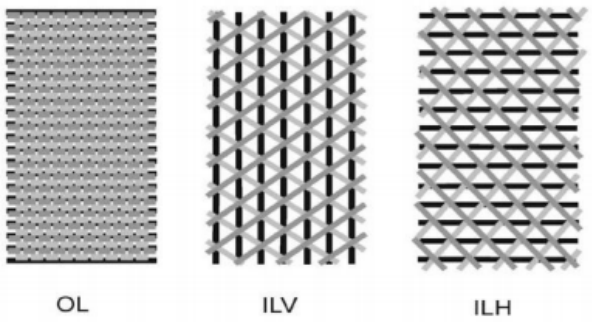

Figure 2.8 Alignment of filaments in scaffold specimens with a 0/60/120 lay-down pattern. In the out of layer (OL) orientation and both in-layer-vertical (ILV) and in-layer-horizontal (ILH) orientations [5]

The inkjet printer or liquid jetting is widely used technology for constructing the soft scaffolds, inkjet bio-printing is most suits for the liquid materials whose viscosities are varying from low to medium [21] [23]. These methods are also called as the drop on demand methods. These are briefly described in the section 2.5. Every technique or method discussed above are having advantages and need not to say disadvantages also. The advantages and disadvantages of the above methods are discussed in the Table 2.1.

Table 2.1 Techniques used in scaffold fabrication with materials, advantages and disadvantages

Technique	Materials	Advantage	Disadvantage	References
Laser sintering	Metals, ceramics, polymers.	Good mechanical strength, Broad range of bulk materials	Energy input, Uncontrolled porosity	[16] [17] [18]
Stereolithography	Reactive resins	Good mechanical strength	Limited to reactive resins (mostly toxic)	[20] [21]
FDM	Thermoplastic polymers	Low costs	Elevated temperatures, Small range of materials, Medium accuracy	[21] [22]
3D plotting	Hydrogels, thermoplastic polymers, resins	Broad range of materials, Incorporation of cells, proteins and fillers	Slow processing, Low accuracy, No standard condition	[21] [23]
Inkjet printing	Wax & wax compounds, liquids.	Excellent accuracy	Slow process, Material limited to low melting point wax	[26] [27]

2.4 Biomaterials for Scaffold

Typically, biomaterials used in the scaffold fabrication are divided into groups, ceramics, synthetic polymers, natural polymers and combination of these [2]. Ceramics are used for the hard scaffold fabrication while other can be used for both hard as well as soft scaffold fabrication. Each of the biomaterials are having their own merits as well as demerits and selection of material dependent on the application and technique.

2.4.1 Biomaterials for hard scaffold

Ceramics like hydroxyapatite (HA) and tri-calcium phosphate (TCP) are generally used for the bone reaeration application, as they possess high stiffness, low elasticity and high brittleness. But their applications become limited because of their high brittleness because they are difficult for shaping for implantation since they cannot sustain the loading for remolding.

There are number of synthetic polymers have been tried to produce the TEC including poly-lactic acid (PLA,) poly-l-lactic acid (PLLA), polystyrene, poly-glycolic acid (PGA) and poly-dl-lactic-co-glycolic acid (PLGA) [2]. These polymers had shown some success since they can be fabricated with controlled architecture and also degradation characteristics can be easily controlled, but they may get rejected cause of reduced bioactivity, also degradation done by hydrolysis has byproduct carbon dioxide which may causes lowering pH and tissue or cell neurosis [24] [25].

2.4.2 Biomaterials for soft scaffold

Hydrogels are also most commonly used jelly like biomaterials, developed by crosslinking structure, containing high water content can be processed under cell friendly conditions and looks more fascinating biomaterials for incorporating the living cells and bioactive component as they can furnish an instructive, aqueous environment, simulating natural extracellular matrix [26] [27]. The most commonly used natural hydrogels are collagen, alginate, agarose, gelatin and chitosan. These

naturally derived hydrogels provide the highest cell viability and proliferation rates because of the presence of the abundant chemical signals. But because of their different printability, construction of the scaffolds with these materials become the challenging [28]. The collagen is having low swelling, good cell survival but its printability, and biocompatibility is not as good [29]. While alginate is having high biocompatibility and printability, it possesses good hydrophilic property but its swelling high and cell survival in alginate is low [30]. Chitosan which printed in pH (basis) is having better biocompatibility but its printability is low [29]. In contrast to natural hydrogels, the synthetically prepared hydrogels like poly (ethylene glycol) and poloxamer are having the better printability [31]. However, they are providing the inert environment to the living cells and hence resulting into the low viability and proliferation rates [26]. In order to deal with these difficulties bio-active compounds like peptide sequences and growth factors are added into synthetic hydrogels to use them in biofabrication [32].

Although considerable progress has been made in the bio-fabrication with both natural as well as synthetic hydrogels, they still have significant difficulties in fulfilling the biological and physical requirements, like complex architecture, mechanical integrity and vibrational degradation rates with respect to time, in order to facilitate cell migration, exit to degradation byproducts, differentiation among original cells and embedded cells and proliferation [2] [16].

2.5 Drop on Demand (DoD) Techniques.

For soft scaffold fabrication the inkjet or liquid jetting technique is used mostly which is nothing but the drop on demand (DoD) technique, Figure 2.9 shows the principle of DoD method, in which the drop of the hydrogel is dropped by actuating the liquid on the crosslinking agent. Here the drop gets polymerized. In the same manner the layer of the hydrogel printed on the bed, bed is then moved down to print the next layer on the top of first layer.

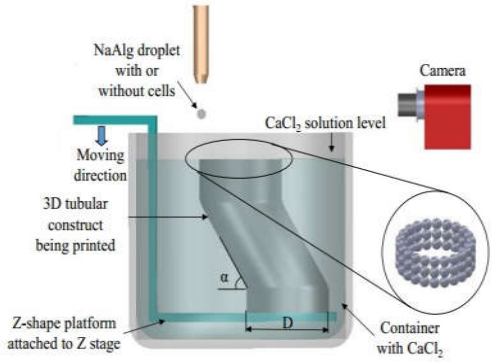

Figure 2.9 Drop on Demand (DoD) method for soft scaffold fabrication [27]

There are many DoD techniques in the area of TE which use hydrogels as a printing material and can be broadly classified as (i) Laser-induced forward transfer, (ii) Inkjet printing & (iii) Robotic dispensing. Figure 2.10, Shows the broad classification of hydrogel based AM techniques [16] [27]. In the laser-based system, focused laser pulses induced on donor slide causes local evaporation of absorbing layer resulting in high gas pressure which propels bioink from another side. These systems provide better resolutions with high gelation rate but fabrication speeds are low [33]. The inkjet printing, in which the bioinks dispense through micro dispensing tips and small droplets are positioned precisely can be further subdivided into the three types based on the actuation methods [34] viz., (i) Electromechanical in which biomaterials are actuated with a piezoelectric material (ii) Electrothermal in which bio inks are thermally actuated. (iii) Electrostatic spraying in which bio inks actuated with voltage difference [35]. The Robotic dispensing approach to is also subdivided into the following three subcategories: (i) Pneumatic (ii) Piston & (iii) screw actuated. Generally, in the robotic dispensing system, to maintain the construct shape, more hydrogel is yielded instead of positioning single droplet [27]. Each of these methods are having advantages & disadvantages.

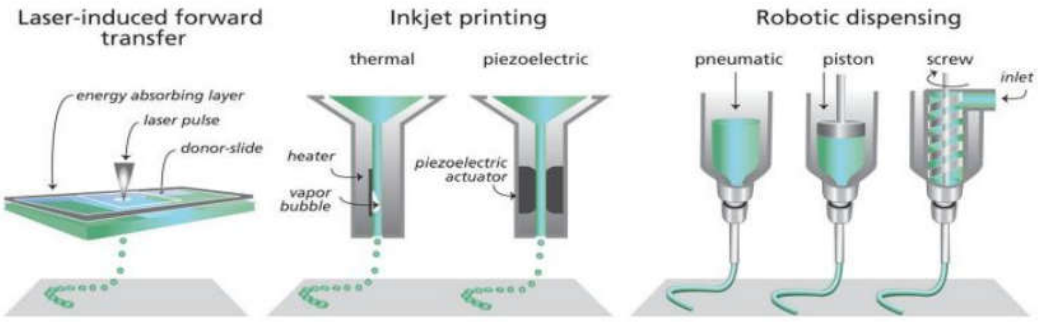

Figure 2.10 Classification & working methodology of the droplet based AM techniques [27]

2.6 Selection of Hydrogels for Droplet-Based Fabrication

The selection of the hydrogel for any specific fabrication method mainly relies on the physicochemical properties of the hydrogels under the conditions imposed by that method. The major properties which decide the printability of the bioink for the specific process are rheological properties such as viscosity, pseudo-plasticity, yield stress. Viscosity is the resistance to fluid flow when the pressure is applied. High viscosity disrupt the droplet formation by surface tension also as the concentration of the solution has been increased, creates prohibitory environment for the tissue formation, cell proliferation and migration. Increased viscosity increases the pressure required for flow which may be harmful for living cells and also cell viability may also affect negatively. Pseudo-plasticity also referred as the shear thinning implies the non-Newtonian behavior of the fluid in which as shear rate increases the viscosity decreases. In the robot dispensing techniques this phenomenon is predominantly observed as the liquid passing through the nozzle the viscosity decreases and when it comes out of the nozzle there is sudden increase in the viscosity which improves the printability [27] [36] [37].

The crosslinking mechanisms like ionic, chemical, thermal, enzymatic, photo-polymerization crosslinking also need to consider while selecting the hydrogel for the

Table 2.2 Hydrogels used in droplet based tissue construction techniques

Hydrogel	Fabrication technique	Gelation method	Remark	References
Alginate	Laser-induced	Ionic	High viability, Medium to high printability	[33] [38]
	Electromechanical	ionic	Good printability, High viability	[39] [40] [35] [41]
	Electrothermal			
	Electrostatic			
	Piston-driven			
	Pneumatic-driven			
Gelatin	Electromechanical	Ionic	Low printability with less viscosities, poor cell differentiation	[17]
	Electrostatic	Chemical		[35]
	Piston-drive	thermal+chemical		[17]
	Pneumatic-driven	pH+themal		[42]
Collagen type	Electrothermal	ionic+enzymatic	Migrating cells	[27] [43]
	Pneumatic-driven	Thermal		
Agar	Piston-drive	Thermal	Good printability	[27]
	Pneumatic-driven	Thermal		
Agarose	Piston-drive	Thermal	Good viability	[26] [44]
	Pneumatic-driven	Thermal		
Alginate+fibrin	Pneumatic-driven	ionic+enzymatic	Poor printability	[27]
Alginate+gelatin	Piston-drive	ionic+thermal	High printability, good viability	[45]
Alginate+gelatin +chitosan	Piston-driven	ionic+chemical + enzymatic	Proliferating cells	[44]

particular technique [4]. Physical crosslinking which further can divide ionic, Stereocomplex and thermal crosslinking mainly rely on non-chemical interactions, which are based on entanglements of polymer chains, ionic or hydrophobic interactions or hydrogen bridges. These are having advantages like excellent biocompatibility with systems and constant viscosity during the deposition but the

structures printed with these crosslinking mechanism are mechanically weak also requires the post processing. With chemical crosslinking one can get the mechanically stable construct also with this post processing of the physically cross-linked construct is possible but controlling the triggered reactivity to control the crosslinking kinetics is difficult [16]. These properties also affect the cell viabilities & proliferations. Table 2.2 shows the different hydrogels used for different droplet-based construction techniques, with mixing different bioinks, droplet-based as well as continuous hydrogel depositions were carried out.

2.7 Summary

In the literature survey, the conventional methods used for in the Tissue Engineering for the fabrication of the scaffold are studied and then they are narrowed down to the AM techniques. The AM techniques further narrowed to the droplet based AM techniques. The survey includes the deep study of the biomaterials used for fabrication of the scaffolds. The material i.e. hydrogel selection of criterion also studied in the literature survey.

Chapter 3

Experimental Setup

3.1 Overview

This chapter describes the experimental setup used in this study. The initial machine and modified machine setup with their specifications, software tools used for the modeling as well as path planning for the study, parameters affecting the deposition and their control and how solutions were prepared has been discussed in detail in the following sections.

3.2 Liquid Dispensing System

In current study, a Fused Deposition Modeling (FDM) machine of AlfaRod AR 1, ALFATEK systems shown in Figure 3.1 is used as the starting point for the development of the new drop on demand methodology. The AlfaRod AR1 is based open source design equipped with infra-red sensors, Ethernet, advanced Arduino micro-controller. This machine gives features like auto-bed leveling, orthogonal compensation, and planarity compensation. This FDM machine can build the 3D model by depositing the small extruded string of molten material layer by layer, which is extruded through the small nozzle. The machine adopts the same G-code format popular in CNC machining. There is wire feed mechanism which pushes the extrusion material in controlled manner. The nozzle is preheated to their glass

transition temperature to melt extrusion materials. The machine can build the volume 200 mm X 200 mm X 200 mm. The maximum bed heating is possible up to 125^0C while maximum nozzle heating can be done till 250^0C.

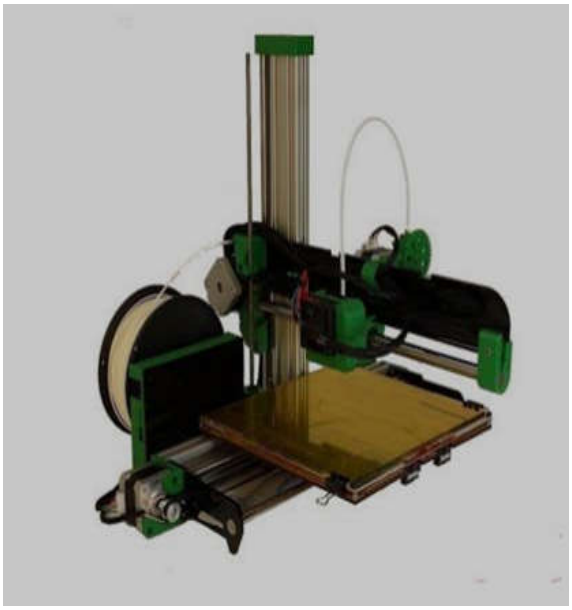

Figure 3.1 AlfaRod AR1, Thermoplastic wire extruder

The AlfaRod AR1 is modified into liquid dispensing system which works on the drop on demand technique for fabrication of the soft tissue hydrogel shown in the Figure 3.2. The wire extruding mechanism of machine is replaced by the special needle-syringe arrangement which is driven by the stepper motor through M5 lead screw. The plunger of the syringe is connected to the pushing/ pulling part of machine whose motion is controlled by the lead screw. The syringe attached to the mechanism is of 5 m barrel in which dispensing biomaterials are filled. Different types of needles or dispensing tips used in current studies are (1) taper ended straight needles (Gauge 24 & 26), (2) blunt ended straight needles (Gauge 24 & 26), (3) Blunt ended tapered tips (Gauge 22).

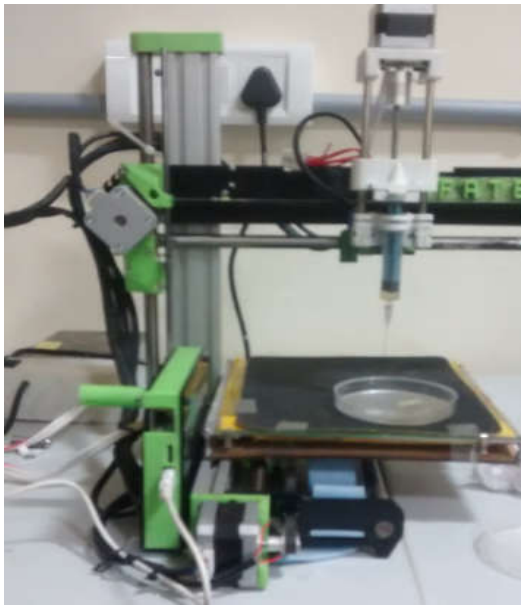

Figure 3.2 Modified AlfaRod AR1 with syringe arrangement

The biomaterials dispensed through the needles on the polymerizing agents are either collected (dropped on $CaCl_2$) in Borosil 3165072 Culture, Petri & S-Line Dishes (Figure 3.3) or in the Borosil 3140077 Crystallizing dishes (Figure 3.4) which were kept on a bed of the machine.

Figure 3.3 Borosil 3165072 Culture, Petri & S-Line Dishes

Figure 3.4 Borosil 3140077 Crystallizing dish.

3.3 Software for Path Planning

As with any other standard 3D printer, the .stl format is the input CAD format for the slicing software. This then to create .gcode file which is consisting of the G codes and can be taken and proceeded as the input file by 3D printer. There are number of CAD packages available, like ProE, CATIA, SolidEdge, SolidWork and many more. The open source slicing software are also available like Slic3R, Kisslicer, Cura, Skeinforge.

In this study the PTC-CreO2.0 (ProE) CAD Package is used to model the part, for processing the .stl file to generate G code file, Slic3R slicing software is used. The configuration setting of the software are given in Table 3.1. The machine path generated G-code is visualed with the help of CIMCO software, where further modification of the file for controlling the top speed of axis and editing the path made can also be carried out. This gcode file can then be imported in Alfaro software to print.

Table 3.1 Slic3R configuration setting used

Print Settings	
Layer Height	0.3 mm
Perimeter	1
Top Layer	0
Bottom Layer	0
Skirt	0
Speed Parameter	
Perimeter	6-35 mm/sec
Infill	6-35 mm/sec
Non print move	5-12 mm/sec
Filament settings	
Diameter	1-3 mm
Extrusion multiplier	1-4.5

3.4 Control Parameters

Selection of suitable optimal control parameters is key for building soft scaffold. Since the DoD method is used the accuracy of dropping layers is greatly affecting by the inertial forces. Also, the controlling flow of liquid and speed of the axes are having critical importance. The polymerized sphere size also depends upon the concentration of the solution, feed rates. For the extrusion of 1 ml volume of liquid, modified machine requires extrusion value as 72 mm.

3.4.1 Flow rate

The plunger movement of the syringe is controlled trough the lead screw and stepper motor. The flow rate can be controlled directly giving the desired value in the gcode file or indirectly though the control of printing speeds.

Though the feed rate is not affecting the droplet size up to a particular range (which keeps accumulating near the nozzle till being big enough to detact), it greatly affects the solidity of the construct in the multi-layer deposition. The higher feed rate would have cause the same amount of bio ink, which would be controlled by the extrusion multiplier, in for shorter time and that would cause the uneven or deformed spheres. Feed rate is independent on the concentration of the solution, with very high feed rates it is possible to reduce the droplet size remarkably, as the liquid comes out as a jet but the controlling droplet size and simultaneously positioning the drops will become more challenging.

The extrusion multiplier factor changes the amount of the flow proportionally. As the machine is modified from the FDM into liquid extrusion machine, this factor is a convenient way to control amount of liquid need to dispense at any particular point.

3.4.2 Print speed

As the polymerized sphere is floating over the $CaCl_2$ the intermediate speedy motion of the bed causes the movement of the sphere. Various printing speeds were

tried to avoid the above problems in addition to the jerk created as a result. Based on trials, 300 mm/min was found to be suitable.

3.4.3 Concentration of solutions

The size of the droplet formed is dependent upon the concentration of the Na-Alg. Increasing the concentration of the Na-Alg will increase the density, which in turn will also increase the viscosity and the resulting droplet size this lowering the resolution. The number of variation of the concentrations of Na-Alg and $CaCl_2$ for better spherical shape. The variation in size and solidity of sphere is varying and discussed in the subsequent chapters.

3.4.4 Kinematic description settings to control floating

Non-printing movements of the axis are at the maximum speed this gives the jerk to the polymerized sphere. The reduction in the speed is ultimately increasing accuracy also provide the sufficient time for gelation or crosslinking. In these studies, non-printing movements tried 300 mm/min to 800 mm/min.

3.5 Solution Preparation

The different concentration by weights of the Na-Alg and the $CaCl_2$ are prepared in triple distilled water. The pure Na-Alg used is of Sisco Research Laboratories Pvt. Ltd. And the $CaCl_2$ (fused) is of SD Fine-Chem limited. The triple distilled water is used which is obtained though PURELAB Option machine of the Labindia Instruments Pvt. Ltd. The weighing machine is used a digital scale of capacity 100 gm with 0.01 gm of increments.

The solutions were always prepared freshly before conducting experiments. The Na-Alg takes time to dissolve in water and hence to accelerate the mixing the solution is heated to 50-55^{0}C and stirred well to ensure a homogeneous mixture. The reaction of the $CaCl_2$ with water is exothermic reaction, it dissolves in water very quickly. But if the solution of $CaCl_2$ kept idle for a long time it starts to settle down

to the bottom and hence needs continuous stirring. Cleaning of dishes used to store $CaCl_2$ is troublesome.

3.6 Summary

This chapter describes how the open source configuration based commercial machine is modified into the liquid dispensing system and proposed to use for the development of the new methodology of soft scaffold fabrication. This includes the modeling of part, path panning, software tools for path planning factors which will affect the scaffold properties and how to control them.

Chapter 4

Studies on Single-Layer Deposition

4.1 Overview

This chapter presents the various studies carried out to develop the methodology and observations of the studies. Initial experiments carried by extruding $CaCl_2$ through syringe into a Na-Alg solution were presented in the first part. Second part describes the reverse and their concentration effects. The different methods tried to reduce the droplets size were also presented in the subsequent sections.

4.2 Extrusion of Calcium Chloride

As per previous studies, have took the $CaCl_2$ in the syringe and it was extruded in circular way over the Na-Alg with different concentrations of both solutions. As $CaCl_2$ was the polymerizing agent, it would start polymerizing the complete Na-Alg solution taken in the dish. If this method would be chosen, then it would become very difficult to print subsequent layers over it as $CaCl_2$ was promoting the crosslinking, after deposition of first layer whatever $CaCl_2$ would remain into Na-

Alg would be utilized as polymerizing agent and whole structure will become solid. Controlling the porosity, which would be one of the important criteria in TE would become very difficult. The continuous polymerization of Na-Alg was termed as the swelling, which was also supported by literature [45]. With this method one cannot proceed for the fabrication of the scaffold since the basic requirement i.e. porosity was vanishing in 3D printing of scaffold with this method.

To observe the swelling effect 10 % $CaCl_2$ was dropped into the 3% and 5% Na-Alg and the images were taken after 5 minute intervals. Figure 4.1 shows swelling effect observed when 10% $CaCl_2$ was dropped into 3% Na-Alg, and Figure 4.2 Swelling effect when 10% $CaCl_2$ was dropped into 5% Na-Alg.

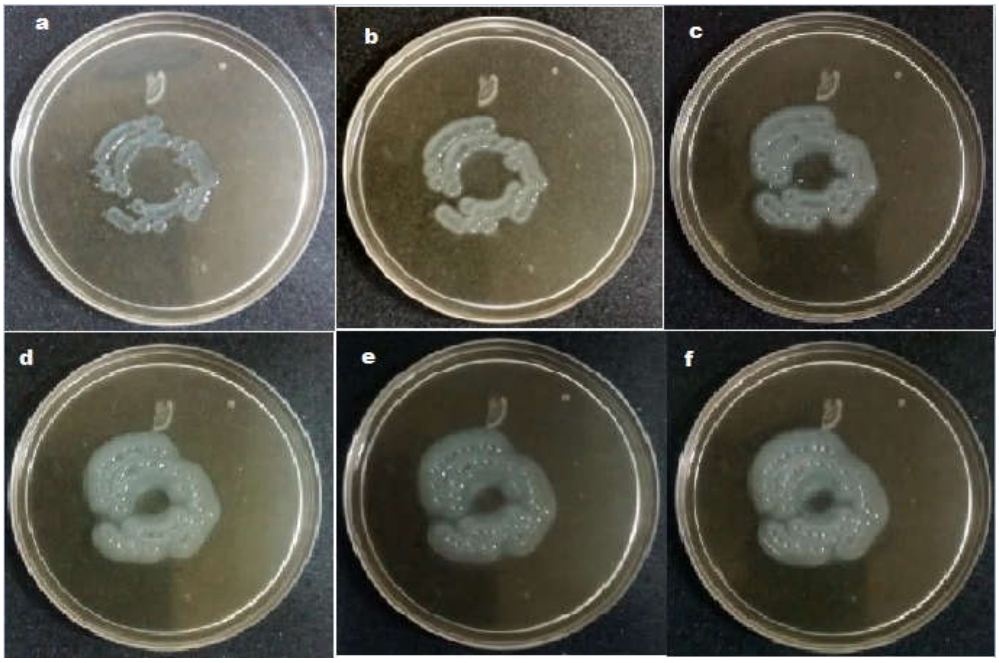

Figure 4.1 Swelling effect, when 10% $CaCl_2$ was dropped into 3% Na-Alg, (a) After 1min. (b) After 10 min, (c) After 30 min, (d) After 1hr, (e) After 2 hr, (f) After 3hr

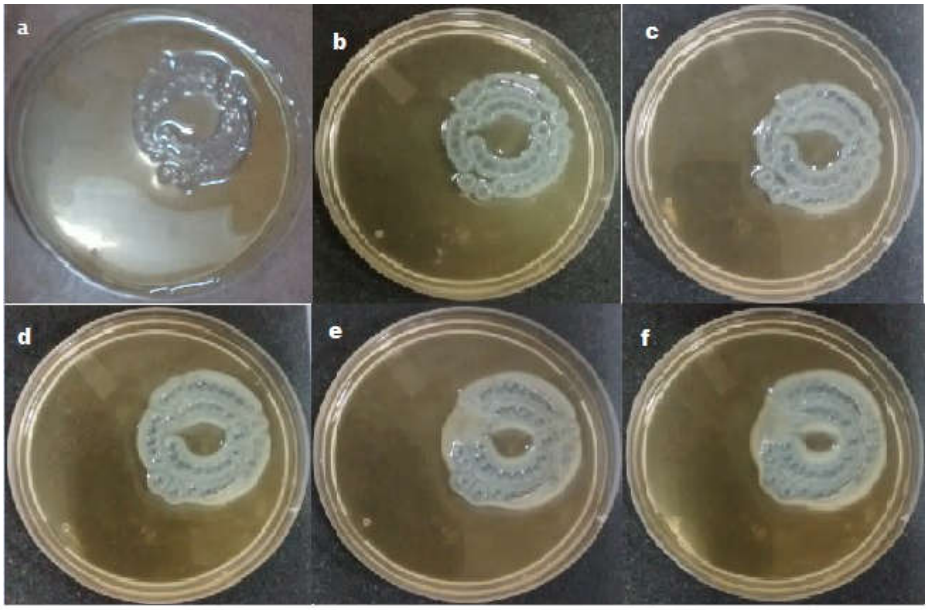

Figure 4.2 Swelling effect when 10% $CaCl_2$ was dropped into 5 % Na-Alg, (a) After 1min, (b) After 10 min, (c) After 30 min, (d) After 1hr (e) After 2 hr, (f) After 3hr

4.3 Extrusion of Sodium-Alginate

To overcome this problem, one has to flip the solutions, ie., Na-Alg was taken in the syringe and $CaCl_2$ was filled in the container. When Na-Alg was dispensed through the needle the polymerizing agent $CaCl_2$ reacted with it, forms the Ca-Alginate and since there would not be any more Na-Alg was available the polymerization process ends with polymerized granules. With this change, it was possible to go for the multi-layer deposition with satisfying the basic requirements of the scaffold.

Different concentrations of Na-Alg (w/v) prepared and tested by taking into syringe were 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5% & 5%. Different concentrations of the $CaCl_2$ which was taken into the container used were 5%, 6%, 7%, 8%, 9% &10%. Effect of concentrations of both Na-Alg and $CaCl_2$ were studied and presented in following subsections. These includes the variation in number of droplets formed, formability and defects. Two considerations were taken into account while measuring the formability viz., (1) The perfectness of sphere excluding the defect and (2) Solidity of the grain. A small tail like defect were also observed in this studies.

4.3.1 Effect of sodium-alginate concentration.

Na-Alg was taken into the syringe and extruded for 1 ml i.e. by 72 mm with extrusion rate 95 mm/min, and the number of droplets were counted with different concentration. Table 4.1 And Table 4.2 shows the variation in number of droplets with variation of concentration. As the concentration of the Na-Alg was increasing the density of solution was increasing which results into increasing diameter of the spheres. With low concentration of the Na-Alg, 2% (w/v), more deformation in shape of grains were observed, while with 5% (w/v) of Na-Alg the tail like deformations were occurred. Table 4.1 lists the droplet geometry for various concentrations of Na-Alg.

Table 4.1 Effect of variation Na-Alg concentration

Na-Alg concentration % (w/v)	No. of droplets 0.4 mm dia. Needle	Volume of each droplet (x 10^{-3} ml)	Diameter of the sphere after polymerization (Approximate ±0.2)
2.0	132±4	7.57	2.2
2.5	127±3	7.87	2.4
3.0	123±3	8.13	2.6
3.5	118±4	8.47	2.6
4.0	111±4	9.00	2.8
4.5	108±3	9.26	3.0
5.0	104±4	9.62	3.0

4.3.2 Effect of calcium chloride concentration

The $CaCl_2$ was used as the crosslinking agent for polymerization of the Na-Alg. For low concentration of $CaCl_2$, the deformations of the polymerized granules were observed to be larger. With increasing concentration of the $CaCl_2$ the spheres formed were stronger and the polymerization with $CaCl_2$ concentration was quicker. Table 4.2 shows the effect of the concentration of Na-Alg and the $CaCl_2$ on the shape of solid grain formed. It was observed that the formability increased with increasing the concentration of the $CaCl_2$, but defects were also increasing. Formability and defects were measured visually over scale of 1 to 3, where 1 indicates poor quality

(less formability and more defects) and 3 indicates the good quality (more formability and less defects).

Figure 4.3 shows the polymerized grains for reference of formability, and defects with different concentrations of Na-Alg and $CaCl_2$ for reference.

Table 4.2 Effects of concentrations of the Na-Alg and $CaCl_2$

Na-Alg % (w/v)	**$CaCl_2$ % (w/v)**	**Formability** **1=very less/unsuccessful** **2= Less** **3= Good**	**Tail defects** **1= More** **2= Medium** **3= Less**
2.0	5	1	3
2.0	6	1	3
2.0	7	1	3
2.0	8	2	3
2.0	9	2	2
2.0	10	2	2
2.5	5	1	3
2.5	6	1	3
2.5	7	2	2
2.5	8	2	2
2.5	9	2	2
2.5	10	2	2
3.0	5	1	3
3.0	6	2	3
3.0	7	2	3
3.0	8	3	3
3.0	9	3	3
3.0	10	3	2
3.5	5	2	3
3.5	6	2	3
3.5	7	2	2
3.5	8	3	2
3.5	9	3	2
3.5	10	3	1
4.0	5	2	2
4.0	6	2	2
4.0	7	2	2

4.0	8	3	1
4.0	9	3	1
4.0	10	3	1
4.5	5	2	2
4.5	6	2	2
4.5	7	3	1
4.5	8	3	1
4.5	9	3	1
4.5	10	3	1
5.0	5	2	2
5.0	6	2	2
5.0	7	3	1
5.0	8	3	1
5.0	9	3	1
5.0	10	3	1

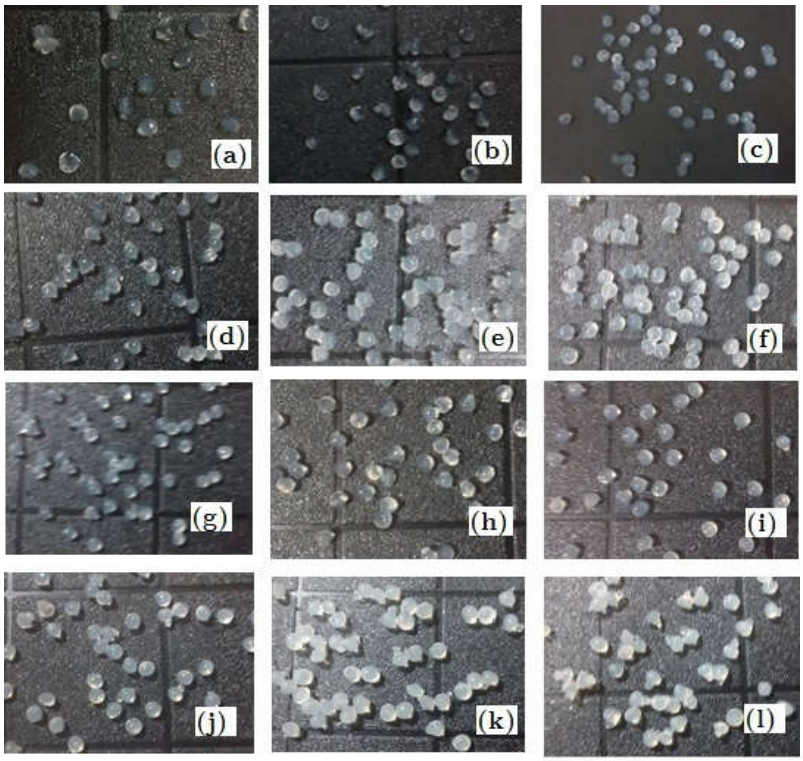

Figure 4.3 Polymerized granules (formability, defects) (a)2%Na-Alg+5%$CaCl_2$ (1, 3). (b)2%Na-Alg+10%$CaCl_2$ (2, 2), (c)2.5%Na-Alg+5%$CaCl_2$ (1, 3), (d)2.5%Na-Alg+10%$CaCl_2$ (2,2), (e)3%Na-Alg+5%$CaCl_2$ (1, 3), (f)3%Na-Alg+9%$CaCl_2$ (3, 3), (g)3.5%Na-Alg+5%$CaCl_2$ (2, 3), (h)3.5% Na-Alg+9%$CaCl_2$ (3,1), (i)4%Na-Alg+5%$CaCl_2$ (2, 2), (j)4%Na-Alg+10%$CaCl_2$ (3, 1), (k) 5%Na-Alg+5%$CaCl_2$ (2, 2), (l) 5%Na-Alg+9%$CaCl_2$ (3, 1)

4.4 Induced Vibration of the Nozzle

As the droplet was detaching from the nozzle only by gravity and surface tension, the size of the droplets was considerably large. Further reduction was needed for accurate and precise realization of the scaffold for TE. Hence to reduce the size of the grains, vibration assisted deposition was tried. The vibration motor of the 6 mm diameter, 12.3 mm in length and 2.1 gm weight was attached to syringe at different positions. The rated vibrations speed of the motor was 9000 rpm with normalized vibration amplitude 1 G. The distance of motor placed was measured from the tip of the needle. When the vibration motor was attached at 30 mm from tip at joint of syringe-needle and 1 ml Na-Alg was dispensed at 95 mm/min the solution was getting spread over bed. The vibrating motor was then attached at 45 mm (Figure 4.4-a), 55 mm (Figure 4.4-b) and 65 mm from tip. But no significant improvement was observed. At distance 45mm from the tip, number of droplets increased slightly as shown in Table 4.3**.** But this marginal improvement came at the cost of low accuracy due to the disturbance of the needles.

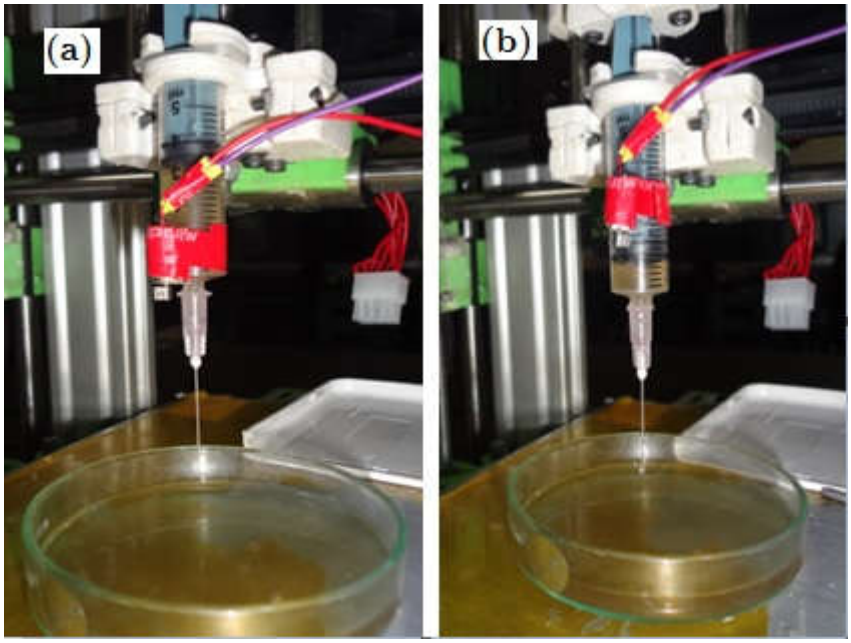

Figure 4.4 Vibration assisted printing, Vibration motor was attached at height (H) from the tip of the needle, (a) H= 45 mm (b) H= 55 mm

Table 4.3 Number of droplets without vibration and with vibration by attaching the vibration motor at different height (H)

Na-Alg Concentration % (w/v)	Droplets without vibration	Droplets without vibration		
		H=45 mm	H=55 mm	H=65 mm
2.0	132±4	136±6	134±4	133±4
2.5	127±3	130±6	127±3	127±3
3.0	123±3	127±5	124±2	123±1
3.5	118±4	121±7	118±3	118±2
4.0	111±4	113±6	111±2	111±1
4.5	108±3	110±7	107±2	108±2
5.0	104±4	107±7	105±2	104±2

4.5 Effect of Needle/Tip Diameter

As discussed in the previous section, the induced vibration of the syringe did not have a significant effect in reducing the size of the polymerized grains. Subsequently, changing the size and shape needle was explored. The following were the three configurations studied: (1) tapered ended straight needle of Gauge 24, (2) tapered ended straight needle of Gauge 26 and (3) blunt ended tapered tip. Gauge 24 needle was having diameter 0.311 mm. 26-Gauge needle was having the inner diameter 0.260 mm and tapered dispensing tip was having inner diameter 0.41 mm. 1 ml liquid was dispensed through each of these needles with the speed of the 95 mm/min. With decreasing diameter, of course the number of drops increases but the variation in number of drops also increases. With blunt ended converging dispensing, the variation in number of drops was decreases remarkably. This was because the straight needles used before were causing more fluid friction and tapered tip, made up of the Teflon was having low friction coefficient. Its converging shape was providing more positional accuracy than the straight needles. The straight needles were chocking very frequently and need replacement, while in the converging tip, even if alginate dried choking the tip, the extruding liquid cleans it automatically.

Table 4.4 shows the number of droplets formed for 1 ml extrusion i.e. 72 mm with the speed of 95 mm/min.

Table 4.4 Effect of variation in diameter of needles or tips

Na-Alg Concentration % (w/v)	Straight needle Gauge 24 I.D.= 0.311 mm		Straight needle Gauge 26 I.D.= 0.260 mm		Tapered Tip Gauge 22 I.D.= 0.410 mm	
	Max	min	Max	Min	Max	Min
2.0	136	128	177	165	68	67
2.5	130	124	169	155	67	66
3.0	127	119	161	148	65	65
3.5	122	114	155	139	63	63
4.0	114	108	146	131	61	61
4.5	112	104	139	124	59	59
5.0	107	101	131	117	58	58

4.6 Single-Layer Deposition

The CAD design of two concentric circle, as shown in the Figure 4.5, was made in PTC-Creo2.0 with outer diameter 35 mm and inner diameter 25 mm. Path was modified in CIMCO, in which the inside deposition path was removed as shown in the Figure 4.5. The printing speeds and feed rates were modified for jerk free movements of the axes. This modified file then run with AlfaRod software, with Na-Alg in the preparing the alginate bed. Figure 4.6 shows the single-layer deposition on alginate bed.

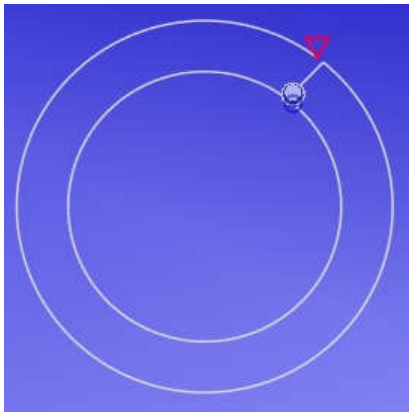

Figure 4.5 Path of deposition for the soft tissue engineering construct

Figure 4.6 Single-layer deposited on the alginate bed

4.7 Summary

Various studies for conducted for realizing single-layer deposition has been presented in this chapter. Of the two possible combinations, extruding the Na-Alg through the syringe into the $CaCl_2$ solution was found to be suitable. Subsequently, the effect of concentration of the solutions was studied. 3% Na-Alg with 8-9% of $CaCl_2$ was found to have the best combination of good formability and least tail defects.

The effect of induced vibration of the needle was also explored, but was found to be insignificant. Use of Teflon made conical nozzle was found to be more suitable than steel parallel needles.

These studies provide the necessary platform for multiple layer deposition, presented in the subsequent chapter.

Chapter 5

Studies on Multi-Layer Deposition

5.1 Overview

Having studied the single-layer deposition, the fabrication of multi-layer scaffold has been explored in this chapter. Usually, in most 3D printing processes, this was just a simple matter with suitable lowering of the platform. However, in the context of DoD techniques, the liquidius nature of the medium makes it a challenging task to have positional control of the detached droplet. Various methods attempted to solve this problem have been presented in this chapter. These were done making use of the parameters obtained from single-layer deposition in the previous chapter.

5.2 Initial Trials

Initially, a cylindrical shape needing 9 layers were printed. This deposition was done with feed rate 900 mm/min and speed of non-printing moves kept at 500 mm/min. After deposition of every layer, manually level of the $CaCl_2$ was downed so that bonding between the initially printed layer and newly printer layer would happen, then after 5 min of curing time, the level of $CaCl_2$ was raided above the newly printed layer such a manner that the cross-linked grains would not float

around. Figure 5.1 shows the multiple layer deposition without alginate bed preparation and Figure 5.2 shows the with alginate bed preparation.

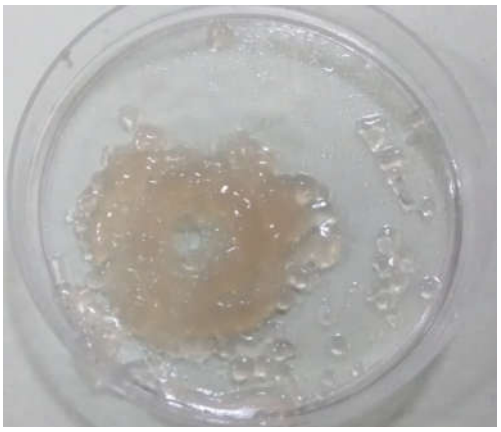

Figure 5.1 Multi-layer deposition without alginate layer preparation

Figure 5.2 Multilayered deposited structure on the alginate layer

5.3 Moving Platform for the Multi-Layer Deposition

For the single-layer deposition, the Borosil 3165072 Culture, Petri & S-Line Dishes were used as the $CaCl_2$ container. But for the multi-layer deposition manually increasing the level of the $CaCl_2$ was disturbing the floating alginate spheres and hence to solve this problem, a moving platform, on which the Na-Alg could be dispensed was created.

5.3.1 Mechanism of the moving platform

The Figure 5.3 shows a mechanism was made up by using the smooth steel rod, M5 lead screw. The other parts were fabricated with the help of U-print 3D printer as well as the Alfatek 3D printer which were made up of ABS plastic, some of them are shown in the Figure 5.4. The Modeling of parts was done in the PTC Creo 2.0. The platform can be driven by the gears which reduced the increment to 0.260 mm per revolution of small gear. The mechanism was designed in such a manner that the bed can remain submerge in the $CaCl_2$, and this can be move in both positive and negative Z direction according to requirement. The mechanism can be fixed to the printer bed.

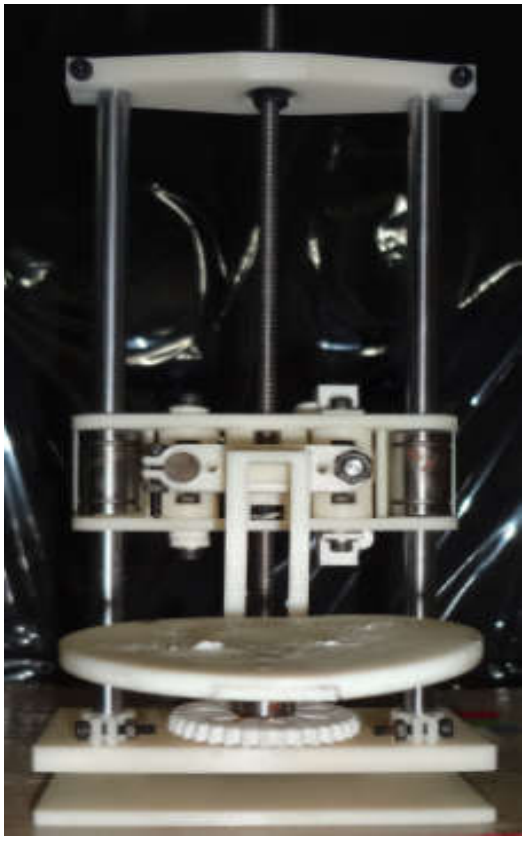

Figure 5.3 Moving bed/platform mechanism for multi-layer deposition

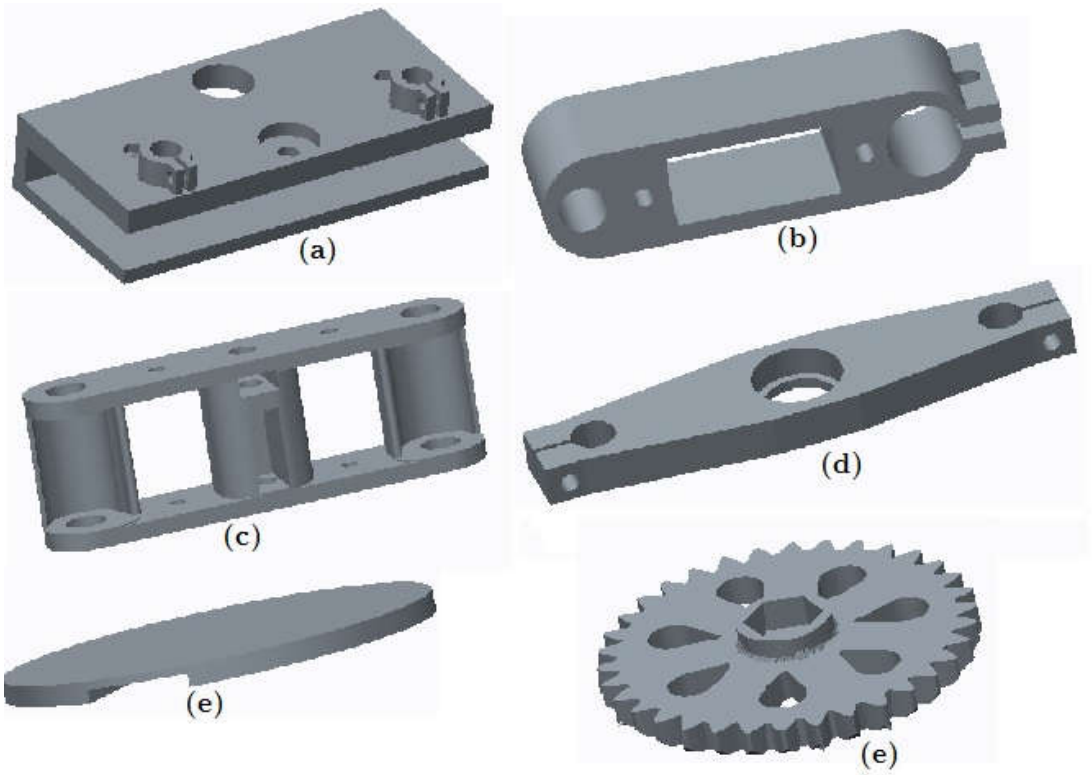

Figure 5.4 CAD models of the parts of the moving platform mechanism

5.3.2 Deposition without alginate layered bed

The 250 ml of 10 % (w/v) concentration $CaCl_2$ would have been taken in the Borosil 3140077 Crystallizing dish. The mechanism was fixed to the bed of the printer. The platform level was adjusted such that it would remain submerged in to the $CaCl_2$ but would not go very deep inside. The depositions were tried with different feed rates varying from 400 mm/min to 2000 mm/min. But since the polymerized grains were not sticking with ABS plastic bed, when bed was moved down grains were floating over instead of moving inside the $CaCl_2$ along with the bed.

5.3.3 Deposition with alginate layered bed

To overcome the floating granules problem, alginate layer was prepared outside on the bed, by spreading the Na-Alg over the bed and then the again the depositions were tried with varying the feed rates from 400 mm/min to 2000 mm/min with keeping the nozzle distance 25 mm from $CaCl_2$ liquid level. Figure 5.5 shows the

printed structure on the alginate layer, deposited at the feed rate 1000 mm/min with wire diameter value as 1 mm and extrusion multiplier value as 3.5 were used.

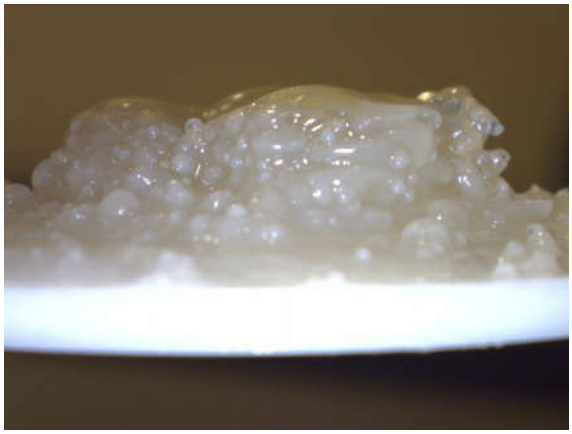

Figure 5.5 Multi-layer deposition on moving platform with alginate layered bed

5.3.4 Deposition with vertical needles

While the moving platform could provide some advantage in controlling the mixing and polymerization of the layers, it could not improve the positional accuracy of the process. As the solidification process was not instantaneous, the extruded liquid tends to scattering in the solution. Hence, maintain the location of the scaffold with the help of positional needles was explored. The vertical needle arrangement comprising of the 24 Gauge needles as shown in the Figure 5.6 was used. Different combinations of these vertical arrangements were tried and finally alginate was printed on the arrangement shown in the Figure 5.8.

It may be noted that at high feed rates, the granules size decrease significantly demanding a very dense needle arrangement. However, at low feed rates although the number of needles was adequate enough to prevent floating, removal of the printed structure from it becomes tedious due to the porosity (and the resulting softness) of the scaffold.

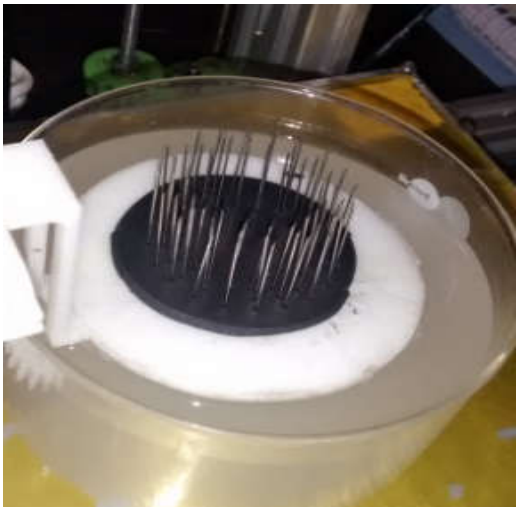

Figure 5.6 Vertical needle arrangement for multi-layer deposition to arrest the floating granules.

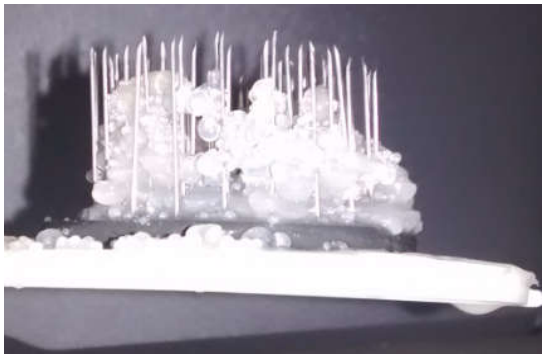

Figure 5.7 Vertical needle arrangement failed with high feed rates.

Figure 5.8 Successful multi-layer deposition with the help of vertical needles arrangement

5.4 Summary

Various challenges associated with multi-layer DoD techniques have been discussed along with some of the attempted techniques to address the same. The floating of the intermediate scaffold, during subsequent deposition can be arrested by suitable selection on the base plate. The main challenge during multi-layer deposition of liquidius medium was ensuring mixing and polymerization occurs only on the top surface. This was implemented with the help of a moving platform. Subsequently, an array of positional needles was also used for improving the locational accuracy of the droplets. These studies gave promising indication of the efficacy of the methods adopted.

Chapter 6

Conclusion and Future Scope

6.1 Conclusions

The droplet based additive manufacturing techniques becomes the popular for the soft scaffold development because of the advantages they provide. This study was to add one more droplet based methodology to economical bio fabrication of soft scaffold, since the hardware used to modify the new liquid dispensing system was very cheap. With further studies it would be possible to achieve the fine matrix structure in low cost. There were following specific conclusions of these studies.

- Swelling phenomenon was more dominating when Na-Alg had been taken into the container and with this methodology it would be very difficult to go for multi-layer deposition, since the crosslinking agent available in the solutions starts the polymerizing newly deposited liquid and make it one structure. For multi-layer deposition the Na-Alg has to be dropped over the crosslinking agent $CaCl_2$, which results into a formation of the polymerized granules and with which the multi-layer deposition would be possible.

- The effects of Na-Alg concentration on the droplet and hence the polymerized grain were studied which was further extended to the effects

of $CaCl_2$ concentration studies and with considering the polymerized grain size, formability and defects the concentrations with surface tension phenomenon the better observations were made at 3-3.5 % Na-Alg (w/v) and 9-10% $CaCl_2$ (w/v).

- Multi-layer depositions were tried with manually lowering and increasing the level of $CaCl_2$ at initial stages and to lower the manual interaction, which was causing disturbance to the granules and to overcome this moving platform was added to the machine which was found very much helpful. The multi-layer deposition was also associated with the controlling the speeds and feed rates as they were majorly affecting the deposition.

6.2 Future Scope

Some of the problems and their possible solutions which can be tried in future are listed below.

- Presently the moving platform was handled manually, which gives the little push or pull to whole mechanism can be isolated by automating it, this will also add the better control over alternate lowering and raising of platform.

- Since the alginate droplets do not wet the ABS plastic, the bio adhesive tape can be applied on the top of moving bed.

- For reduction of droplets the micro dispenser can be used, which can give the micro granules, high precision and accuracy and better control over the dispensing the liquid.

- The independent Z axis can be implemented which can obsolete the jerk to the floating polymerized granules.

- The porosity of the scaffold structure, its strength, cell viability also can be studied in future.

References

[1] *Advanced Manufacturing Technology for Medical Applications: Reverse Engineering, Software Conversion and Rapid Prototyping*. John Wiley & Sons, 2006.

[2] F. O'Brien, "Biomaterials & scaffolds for tissue engineering," *Mater. Today*, vol. 14, no. 3, pp. 88–95, 2011.

[3] I. Martin, D. Wendt, and M. Heberer, "The role of bioreactors in tissue engineering.," *Trends Biotechnol.*, vol. 22, no. 2, pp. 80–6, 2004.

[4] A. Iordanidis, *Mathematical Modeling of Catalytic Fixed Bed Reactors*. 2002.

[5] S. Ansari, "Studies on Additive Manufacturing of Hard and Soft Scaffold for Tissue Engineering Applications," 2015.

[6] S. J. Hollister, "Scaffold engineering: a bridge to where?," *Biofabrication*, vol. 1, no. 1, p. 012001, 2009.

[7] B. Subia, J. Kundu, and S. Kundu, "Biomaterial scaffold fabrication techniques for potential tissue engineering applications," *Tissue Eng.*, no. 3, pp. 141–159, 2010.

[8] A. G. Mikos, G. Sarakinos, S. M. Leite, J. P. Vacant, and R. Langer, "Laminated three-dimensional biodegradable foams for use in tissue engineering," *Biomaterials*, vol. 14, no. 5, pp. 323–330, Apr. 1993.

[9] P. X. Ma and R. Langer, "Fabrication of Biodegradable Polymer Foams for Cell Transplantation and Tissue Engineering," in *Tissue Engineering*, New Jersey: Humana Press, pp. 47–56.

[10] D. W. Hutmacher, "Scaffolds in tissue engineering bone and cartilage,"

Biomaterials, vol. 21, no. 24, pp. 2529–2543, 2000.

[11] Y.-C. Huang and D. Mooney, "Gas Foaming to Fabricate Polymer Scaffolds in Tissue Engineering," in *Scaffolding In Tissue Engineering*, CRC Press, 2005, pp. 155–167.

[12] L. A. Smith, J. A. Beck, P. X. Ma, L. A. Smith, J. A. Beck, and P. X. Ma, "Nanofibrous Scaffolds and their Biological Effects," in *Nanotechnologies for the Life Sciences*, Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA, 2007.

[13] A. Salerno, M. Fern??ndez-Guti??rrez, J. San Rom??n Del Barrio, and C. Domingo, "Bio-safe fabrication of PLA scaffolds for bone tissue engineering by combining phase separation, porogen leaching and scCO2 drying," *J. Supercrit. Fluids*, vol. 97, pp. 238–246, 2015.

[14] L. Lin, S. Ju, L. Cen, H. Zhang, and Q. Hu, "Fabrication of porous β-TCP scaffolds by combination of rapid prototyping and freeze drying technology," in *7th Asian-Pacific Conference on Medical and Biological Engineering*, Berlin, Heidelberg: Springer Berlin Heidelberg, 2008, pp. 88–91.

[15] T. Billiet, M. Vandenhaute, J. Schelfhout, S. Van Vlierberghe, and P. Dubruel, "A review of trends and limitations in hydrogel-rapid prototyping for tissue engineering," *Biomaterials*, vol. 33, no. 26, pp. 6020–6041, 2012.

[16] R. Gauvin, Y.-C. Chen, J. W. Lee, P. Soman, P. Zorlutuna, J. W. Nichol, H. Bae, S. Chen, and A. Khademhosseini, "Microfabrication of complex porous tissue engineering scaffolds using 3D projection stereolithography.," *Biomaterials*, vol. 33, no. 15, pp. 3824–34, May 2012.

[17] T. M. Hsieh, C. W. B. Ng, K. Narayanan, A. C. A. Wan, and J. Y. Ying, "Three-dimensional microstructured tissue scaffolds fabricated by two-photon laser scanning photolithography.," *Biomaterials*, vol. 31, no. 30, pp. 7648–52, Oct. 2010.

[18] M. T. Raimondi, S. M. Eaton, M. Laganà, V. Aprile, M. M. Nava, G. Cerullo, and R. Osellame, "Three-dimensional structural niches engineered via two-

photon laser polymerization promote stem cell homing.," *Acta Biomater.*, vol. 9, no. 1, pp. 4579–84, 2013.

[19] R. Landers, a. Pfister, U. Hübner, H. John, R. Schmelzeisen, and R. Mülhaupt, "Fabrication of soft tissue engineering scaffolds by means of rapid prototyping techniques," *J. Mater. Sci.*, vol. 37, no. 15, pp. 3107–3116, 2002.

[20] T. Serra, J. A. Planell, and M. Navarro, "High-resolution PLA-based composite scaffolds via 3-D printing technology," *Acta Biomater.*, vol. 9, no. 3, pp. 5521–5530, 2013.

[21] W. Lin, "Improvement of 3D Printing Quality for Fabricating Soft Scaffolds," 2015.

[22] S. Yang, K. F. Leong, Z. Du, and C. K. Chua, "The design of scaffolds for use in tissue engineering. Part I. Traditional factors.," *Tissue Eng.*, vol. 7, no. 6, pp. 679–689, 2001.

[23] H. Liu, E. B. Slamovich, and T. J. Webster, "Less harmful acidic degradation of poly(lacticco-glycolic acid) bone tissue engineering scaffolds through titania nanoparticle addition.," *Int. J. Nanomedicine*, vol. 1, no. 4, pp. 541–5, 2006.

[24] N. E. Fedorovich, J. Alblas, J. R. de Wijn, W. E. Hennink, A. J. Verbout, and W. J. A. Dhert, "Hydrogels as Extracellular Matrices for Skeletal Tissue Engineering: State-of-the-Art and Novel Application in Organ Printing," *Tissue Eng.*, vol. 13, no. 8, pp. 1905–1925, Aug. 2007.

[25] J. Malda, J. Visser, F. P. Melchels, T. Jüngst, W. E. Hennink, W. J. A. Dhert, J. Groll, and D. W. Hutmacher, "25th Anniversary Article: Engineering Hydrogels for Biofabrication," *Adv. Mater.*, vol. 25, no. 36, pp. 5011–5028, 2013.

[26] D. Seliktar, "Designing cell-compatible hydrogels for biomedical applications.," *Science*, vol. 336, no. 6085, pp. 1124–8, Jun. 2012.

[27] S. V Murphy, A. Skardal, and A. Atala, "Evaluation of hydrogels for bio-printing applications.," *J. Biomed. Mater. Res. A*, vol. 101, no. 1, pp. 272–84, Jan. 2013.

[28] Y. Zhang, Y. Yu, H. Chen, and I. T. Ozbolat, "Characterization of printable cellular micro-fluidic channels for tissue engineering.," *Biofabrication*, vol. 5, no. 2, p. 025004, 2013.

[29] R. Censi, W. Schuurman, J. Malda, G. di Dato, P. E. Burgisser, W. J. A. Dhert, C. F. van Nostrum, P. di Martino, T. Vermonden, and W. E. Hennink, "A Printable Photopolymerizable Thermosensitive p(HPMAm-lactate)-PEG Hydrogel for Tissue Engineering," *Adv. Funct. Mater.*, vol. 21, no. 10, pp. 1833–1842, May 2011.

[30] C. A. DeForest and K. S. Anseth, "Advances in Bioactive Hydrogels to Probe and Direct Cell Fate," *Annu. Rev. Chem. Biomol. Eng.*, vol. 3, no. 1, pp. 421–444, Jul. 2012.

[31] B. Guillotin, A. Souquet, S. Catros, M. Duocastella, B. Pippenger, S. Bellance, R. Bareille, M. Rémy, L. Bordenave, J. Amédée j, and F. Guillemot, "Laser assisted bioprinting of engineered tissue with high cell density and microscale organization," *Biomaterials*, vol. 31, no. 28, pp. 7250–7256, 2010.

[32] T. Billiet, M. Vandenhaute, J. Schelfhout, S. Van Vlierberghe, and P. Dubruel, "Biomaterials A review of trends and limitations in hydrogel-rapid prototyping for tissue engineering," *Biomaterials*, vol. 33, no. 26, pp. 6020–6041, 2012.

[33] W. Zhang and X. He, "Encapsulation of Living Cells in Small (~100 μm) Alginate Microcapsules by Electrostatic Spraying: A Parametric Study," *J. Biomech. Eng.*, vol. 131, no. 7, p. 074515, 2009.

[34] W. Schuurman, P. A. Levett, M. W. Pot, P. R. van Weeren, W. J. A. Dhert, D. W. Hutmacher, F. P. W. Melchels, T. J. Klein, and J. Malda, "Gelatin-Methacrylamide Hydrogels as Potential Biomaterials for Fabrication of Tissue-Engineered Cartilage Constructs," *Macromol. Biosci.*, vol. 13, no. 5, pp. 551–561, May 2013.

[35] B. A. Aguado, W. Mulyasasmita, J. Su, K. J. Lampe, and S. C. Heilshorn, "Improving Viability of Stem Cells During Syringe Needle Flow Through the Design of Hydrogel Cell Carriers," *Tissue Eng. Part A*, vol. 18, no. 7–8, pp.

806–815, Apr. 2012.

[36] S. Catros, F. Guillemot, A. Nandakumar, S. Ziane, L. Moroni, P. Habibovic, C. van Blitterswijk, B. Rousseau, O. Chassande, J. Amédée, and J.-C. Fricain, "Layer-by-layer tissue microfabrication supports cell proliferation in vitro and in vivo.," *Tissue Eng. Part C. Methods*, vol. 18, no. 1, pp. 62–70, 2012.

[37] T. Boland, X. Tao, B. J. Damon, B. Manley, P. Kesari, S. Jalota, and S. Bhaduri, "Drop-on-demand printing of cells and materials for designer tissue constructs," *Mater. Sci. Eng. C*, vol. 27, no. 3, pp. 372–376, 2007.

[38] K. Arai, S. Iwanaga, H. Toda, C. Genci, Y. Nishiyama, and M. Nakamura, "Three-dimensional inkjet biofabrication based on designed images," *Biofabrication*, vol. 3, no. 3, p. 034113, 2011.

[39] X. Shi, W. Wang, and A. Wang, "PH-responsive sodium alginate-based superporous hydrogel generated by an anionic surfactant micelle templating," *Carbohydr. Polym.*, vol. 94, no. 1, pp. 449–455, 2013.

[40] F. P. W. Melchels, M. A. N. Domingos, T. J. Klein, J. Malda, P. J. Bartolo, and D. W. Hutmacher, "Additive manufacturing of tissues and organs," *Prog. Polym. Sci.*, vol. 37, no. 8, pp. 1079–1104, 2012.

[41] K. Jakab, C. Norotte, B. Damon, F. Marga, A. Neagu, C. L. Besch-Williford, A. Kachurin, K. H. Church, H. Park, V. Mironov, R. Markwald, G. Vunjak-Novakovic, and G. Forgacs, "Tissue Engineering by Self-Assembly of Cells Printed into Topologically Defined Structures," *Tissue Eng. Part A*, vol. 14, no. 3, pp. 413–421, 2008.

[42] M. Neufurth, X. Wang, H. C. Schröder, Q. Feng, B. Diehl-Seifert, T. Ziebart, R. Steffen, S. Wang, and W. E. G. Müller, "Engineering a morphogenetically active hydrogel for bioprinting of bioartificial tissue derived from human osteoblast-like SaOS-2 cells.," *Biomaterials*, vol. 35, no. 31, pp. 8810–9, 2014.

[43] B. Duan, L. A. Hockaday, K. H. Kang, and J. T. Butcher, "3D bioprinting of heterogeneous aortic valve conduits with alginate/gelatin hydrogels.," *J. Biomed. Mater. Res. A*, vol. 101, no. 5, pp. 1255–64, May 2013.

[44] W. Wang and A. Wang, "Synthesis and swelling properties of pH-sensitive semi-IPN superabsorbent hydrogels based on sodium alginate-g-poly(sodium acrylate) and polyvinylpyrrolidone," *Carbohydr. Polym.*, vol. 80, no. 4, pp. 1028–1036, 2010.

[45] T. Boland, T. Xu, B. Damon, and X. Cui, "Application of inkjet printing to tissue engineering.," *Biotechnol. J.*, vol. 1, no. 9, pp. 910–7, 2006.